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Functional expression of arginine kinase improves recovery from pH stress of *Escherichia coli*

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Abstract

Acid stress in *Escherichia coli* involves a complex resource- and energy-consuming response mechanism. By overexpression of arginine kinase from *Limulus polyphemus* in *E. coli*, we improved the recovery from a transient pH stress. While wild type *E. coli* resumed growth after a transient pH reduction to pH 3 for 1 h with a rate that was 25% lower than before the stress, the arginine kinase expressing strain continued to grow as rapidly as before. This effect is presumably caused by the physiological function of arginine kinase as a short term energy buffer in the form of phosphoarginine, but a pH-buffering effect cannot be excluded.

Introduction

Cells of higher organisms often contain so-called phosphagen kinases that serve as transient ATP buffering systems during high or fluctuating energy requirements. Perhaps the most well-known of these is creatine kinase (CK) which is found in all vertebrates, as well as in sponges and in some worms (Ellington 2001). CK isoenzymes exert spatial and temporal energy buffering functions and are involved in intracellular pH homeostasis (Wallimann 1994). Arginine kinase (AK; EC 2.7.3.3) is a conceptually simpler phosphagen kinase and occurs in insects (Grieshaber *et al.* 1994), crustaceans (Wyss & Kaddurah-Daouk 2000) and in certain unicellular organisms (Pereira *et al.* 2000). Although transient ATP buffering is the main function of AK (Ellington 2001), an involvement in pH buffering cannot be excluded. AK uses arginine (Arg) to create a metabolically inert pool of phosphoarginine (PArg), according to the following reaction (Wyss & Kaddurah-Daouk 2000):

$$MgADP^- + PArg^{2-} + H^+ \rightleftarrows mgATP^{2-} + Arg.$$

Although microorganisms are often exposed to rapidly changing environmental conditions and fluctuating availability of energy, phosphagen kinases seem to have evolved only with metazoans (Ellington 2001). Exceptions are the unicellular eukaryotes *Trypanosoma* spp. and *Paramecium caudatum*, probably as the result of horizontal gene transfer (Noguchi *et al.* 2001, Pereira *et al.* 2002). Since the high motility of these latter organisms requires large bursts of energy, the availability of an ATP buffering system obviously conferred a significant evolutionary advantage.

Bacteria frequently encountered environmental stresses that generate a severe demand for ATP (Kobayashi *et al.* 1986), as for example exposure to low pH in the case of *Escherichia coli* (Bearson *et al.* 1997). The most prominent response to pH stress is activation of an ATPase, which compensates variations in cytoplasmic pH by extruding protons to the periplasm, with concomitant consumption of ATP (Kobayashi *et al.* 1986). Moreover, severe intracellular acidification impairs ATP production from fueling pathways as a consequence of enzyme inhibition (Booth *et al.* 2002). Further protective mechanisms include proton extruding ion antiporters and symporters (Booth *et al.* 2002, Epstein & Kim 1971, Zakharyan & Trchounian 2001) and proton-consuming amino acid decarboxylases (Castanie-Cornet *et al.* 1999).

Recently, we showed that heterologous expression of AK in the unicellular yeast *Saccharomyces cere-*

visiae provided a transient ATP buffer, which also helped to overcome a transient, energy-demanding stress of short term starvation (Canonaco *et al*. 2002). Functional expression of creatine kinase was demonstrated in *E. coli* (Koretsky & Traxler 1989) but data from our own laboratory showed that creatine kinase had no apparent beneficial effect on cellular physiology of this organism (Cakar 2000). Since AK expression was successful in yeast, we extend here our studies on transient ATP-buffering systems to prokaryotes by overexpressing AK from *Limulus polyphemus* in *E. coli*.

Materials and methods

Strains and plasmids construction

E. coli wild-type strain MG1655 (λ^-, F^-, *rph-1*) was used for all physiological experiments, and *E. coli* DH5α [F^-/*endA1 hsdR17*(r_k-m_k^+) *glnV44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ*(lacZYA-argF)U169 deoR* (ϕ80*dlac*Δ*(lacZ)M15*)] was used for genetic constructions. The expression vector p*Trc*99A (Pharmacia Biotech) was used for heterologous gene expression. Expression was driven from the strong, IPTG-inducible *trc* promoter. The AK-encoding gene of *L. polyphemus* was PCR-amplified from plasmid pET-22b(+):AK (Strong & Ellington 1995) with the primers 5′-GGAATTCATGGTGGACCAGGCAACATTG-3′ and 5′-TGCGGTCGACTTAGGCAGCAGCCTTTTCCATC-3′. The resulting *Eco*RI/*Sal*I fragment was cloned into p*Trc*99A to yield pAK, which was transformed in *E. coli* by electroporation using a GenePulser (Biorad).

Media and growth conditions

Luria–Bertani (LB) and M9 minimal medium were prepared as described previously (Sauer *et al.* 1999). M9 medium was supplemented with glucose at 5 g l^{-1} and ampicillin at 50 mg l^{-1} for plasmid maintenance. For induction of AK expression, IPTG was added prior to inoculation at the desired concentration. Cultivation was performed aerobically in 500 ml baffled shake flasks with maximally 50 ml medium at 37 °C on a gyratory shaker at 200 rpm.

Transient pH stress experiments were performed by growing cultures to a turbidity at 600 nm (OD_{600}) of about 0.5 in M9 medium with a pH of 6.5, before the pH was reduced to 3 with 10% (v/v) H_3PO_4. After 1 h, pH 6.5 was re-established using 100 mM KOH.

Benzoate and acetate stress experiments were performed by growing cultures to an OD_{600} of about 0.5 in M9 medium, before sodium benzoate or sodium acetate were added to give 2 mM and 8 mM, respectively.

Analytical procedures

Cell growth was monitored by the increase in OD_{600}. Crude cell extracts for determination of AK activity and ATP concentration were prepared from stationary phase cells by 10 to 50-fold concentrated culture aliquots in 0.9% NaCl and 10 mM $MgSO_4$. Cells were then disrupted by sonication at 100 W for 1 min on ice.

For determination of AK activity, samples were frozen at −70 °C and analyzed within a week. AK activity using pH-stat analysis, arginine concentration, and intracellular ATP and ADP concentrations were determined as described previously (Canonaco *et al.* 2002). Intracellular metabolite concentrations were calculated from the experimentally determined concentration using a cellular volume of 3.2 μl mg^{-1} protein.

Determination of physiological parameters

In batch cultures, the exponential growth phase was identified by log-linear regression of OD_{600} versus time, with maximum growth rate (μ_{max}) as the regression coefficient. To calculate specific production rates, OD_{600} values were converted to cellular dry weight (cdw) using a predetermined correlation factor of 0.51 g l^{-1} (cdw) per OD_{600} unit. The biomass concentration was calculated as cdw per volume unit.

Results

Establishing a functional arginine kinase system

Upon transformation of pAK into *E. coli* MG1655, the maximum *in vitro* activity of AK was attained at an IPTG concentration of 100 μm (Figure 1). This value corresponds to about the same level of specific AK activity as found in muscles of *Limulus polyphemus* (Pereira *et al*. 2002).

To determine the impact of Arg feeding on intracellular Arg levels, we grew AK-expressing and control strains at different extracellular Arg concentrations (Figure 2). While the AK-expressing strain always had slightly lower intracellular Arg concentrations, the difference with respect to the control strain became smaller when 10 mM or more Arg were added to the

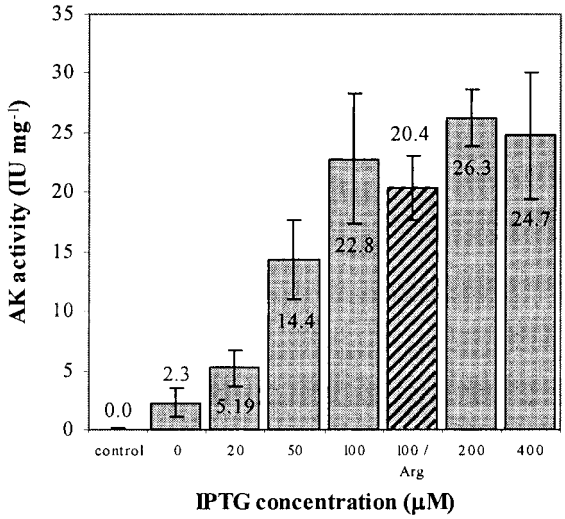

Fig. 1. Specific arginine kinase activity at different levels of IPTG induction. Enzymatic activity was determined in crude cell extracts harvested from stationary phase, pAK carrying *E. coli*. The control is *E. coli* with p*Trc*99A. The hatched bar indicates cells grown in the presence of 10 mM Arg. Error bars indicate the standard deviation from triplicate determinations.

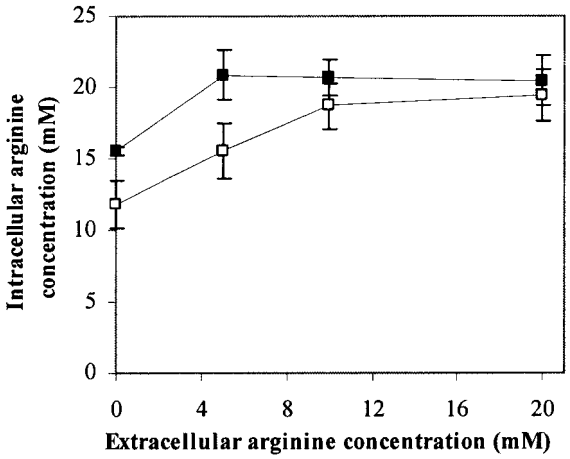

Fig. 2. Intracellular arginine levels at different extracellular arginine concentrations. Intracellular Arg concentrations were determined at different levels of Arg supplementations during growth of *E. coli* MG1655 harboring the control plasmid (■) or pAK (□).

medium. The control strain already reached the maximum intracellular Arg concentration of about 20 mM at 5 mM Arg supplementation, while the AK strain attained the maximum intracellular concentration at 10 mm Arg supplementation.

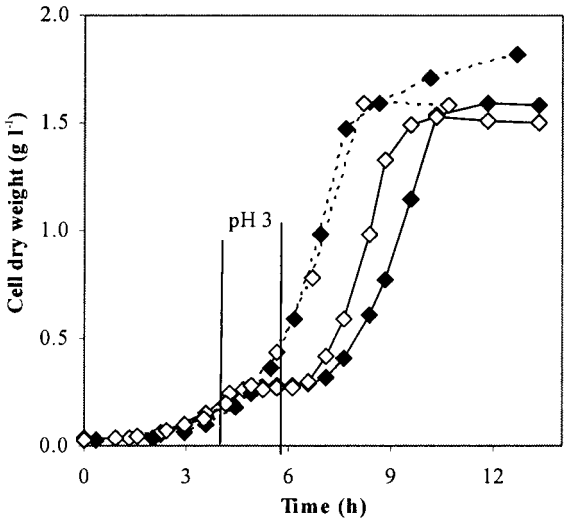

Fig. 3. Impact of a transient medium acidification to pH 3 during growth of AK-expressing (◊) and control (♦) strains. The induction level was 100 μM IPTG and the M9 medium was supplemented with 10 mM arginine. Cultures were either kept at normal pH (pH 6.5; dashed lines) or subjected to transient pH stress (1 h at pH 3; continuous lines). The figure shows one representative of 3 independent experiments performed.

Expression of AK improves recovery after transient pH stress

AK expression had no appreciable influence on the maximum specific growth rate or the biomass yield on glucose during batch growth, when compared to the control (Table 1). In fact, both values were slightly reduced. The presence of 10 mM Arg in the medium increased both μ_{max} and $Y_{(X/S)}$ in control cultures but had little effect on AK expressing cells (Table 2).

Next, we analyzed the potential impact of AK expression during transient pH stress, that was expected to exert an ATP drain among other effects (Bearson *et al.* 1997, Kobayashi *et al.* 1986). Specifically, the pH of exponentially growing cultures (OD_{600} of 0.5) was shifted for 1 h from pH 6.5 to pH 3, and afterwards followed by re-establishing pH 6.5 (Figure 3 and Table 2).

A decrease to pH 3 is well below the value for which the induction of the acid tolerance response is reported (Foster 1999). Indeed, the intracellular ATP level dropped from 5.5 ± 0.2 mM in unstressed cells to 4.2 ± 0.2 M during low-pH stress in the control and the AK-expressing strain (Figure 4). While lag-phases were similar in both strains after the stress, the post-stress growth rates differed significantly. The AK-expressing strain resumed growth at about the

Table 1. AK induction level on growth rate and yield. The standard deviations on the indicated values are from duplicate experiments.

IPTG concentration (mM)	Maximum specific growth rate (h^{-1})	Biomass yield on glucose (g g^{-1})
Control	0.67 ± 0.01	0.36 ± 0.01
0	0.62 ± 0.01	0.35 ± 0.01
50	0.6 ± 0.02	0.33 ± 0
100	0.6 ± 0.03	0.32 ± 0
400	0.54 ± 0.02	0.33 ± 0.01

Table 2. Growth rate and yield before and after a transient pH reduction for 1 h from 6.5 to 3. 'Arg' indicates cultures supplemented with 10 mM arginine. In all cases, the M9 medium was supplemented with 100 μM IPTG. The standard deviations on the indicated values are from duplicate experiments.

Strain	Maximum specific growth rate (h^{-1})	Biomass yield on glucose (g g^{-1})
Before pH stress		
Control	0.61 ± 0.01	0.34 ± 0.02
pAK	0.6 ± 0	0.32 ± 0.01
Control, Arg	0.67 ± 0	0.36 ± 0.01
pAK, Arg	0.56 ± 0.01	0.32 ± 0.01
After pH stress		
Control, Arg	0.51 ± 0	0.32 ± 0
pAK, Arg	0.67 ± 0.01	0.31 ± 0

same rate as observed before the stress, but the control grew significantly slower (Table 2).

Discussion

Previously, we demonstrated an improved resistance to transient pH and starvation stress by metabolic engineering of a functional phosphagen kinase system into the yeast *S. cerevisiae* (Canonaco *et al.* 2002). Although another phosphagen kinase has been functionally expressed in a prokaryote before (Koretsky & Traxler 1989), we show here for the first time a beneficial effect of phosphagen kinase overexpression in a prokaryote; namely improved recovery from a transient pH stress in *E. coli*. Apparently, AK expression has little if any protective effect during the stress, since global intracellular ATP levels were similarly reduced in control and the AK strain. Although both strains resumed growth immediately upon reestablishment of the original pH, the AK-expressing strain attained reproducibly a much higher growth rate than its control. Thus, it appears that AK expression improves recov-

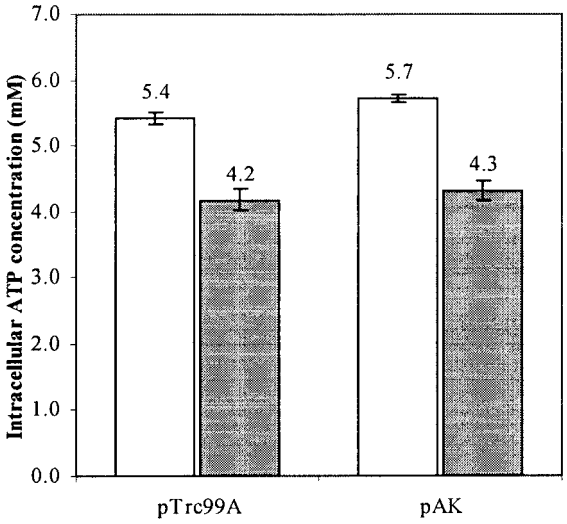

Fig. 4. Intracellular ATP levels during the transient pH stress. Intracellular ATP concentrations were determined in unstressed cultures (white bars) and during transient stress (gray bars) of *E. coli* MG1655 harboring the control plasmid or pAK. The M9 medium was supplemented with 10 mM Arg. The pH of one of the cultures was reduced to 3 and after 1 h a sample was harvested both from stressed and unstressed cultures. Intracellular ATP concentrations were determined as the mean from three independent measurements in two parallel cultures.

ery from a transient stress, which reduced intracellular ATP concentration.

Our study provides indirect evidence for the formation of the storage phosphagen, PArg, since the intracellular Arg concentration was always lower in strains overexpressing AK when compared to the control. Moreover, more than 10 mM extracellular Arg was necessary to achieve maximum intracellular Arg concentrations in the AK expressing strain, while 5 mM was sufficient for the control, presumably because a significant portion of Arg was converted to PArg by the AK reaction.

The beneficial effect of arginine kinase may be related to overcoming an energy-related or a low-pH-related buffering. While pH buffering by arginine kinase was so far not shown explicitly in organisms that express this enzyme naturally, our results would also be consistent with proton scavenging by dephosphorylation of phosphoarginine (see Equation (1)).

The results presented here and in a previous study (Canonaco *et al.* 2002) show that even lower eukaryotes and prokaryotes can be metabolically engineered with transient ATP-buffering systems working in the range of seconds and perhaps minutes. This may be of biotechnological relevance for industrial conditions that are characterized by fluctuating energy demands and availability, as is found for example in large-scale bioreactors with imperfect mixing and thus local zones with oxygen or carbon source limitation (Enfors *et al.* 2001).

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